

The most reliable LC-EC
applications for
Antibiotics analysis

Aminoglycosides

Amikacin
Framycetin Sulphate
Gentamicin Sulphate
Kanamycin Sulphate
Lincomycin
Neomycin
Spectinomycin
Tobramycin

Macrolide antibiotics

Azithromycin
Azaerythromycin
Clarithromycin
Erythromycin
Roxithromycin

Gentamicin Sulphate According to EP and USP Method

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- **European Pharmacopoeia 8.1 (2014)**
 - **U.S. Pharmacopeia 37-NF32 (2014)**
 - **Analysis of composition and impurities**
 - **Reproducible and robust**
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Summary

The Gentamicin sulphate analysis in pharmaceutical preparations was evaluated on an Antec ALEXYS LC-EC analyzer, using the exact method and conditions described in the official 2014 USP monograph (37-NF32) and EP monograph (8.0).

In this application note typical results obtained with the ALEXYS[®] analyzer are reported, demonstrating its performance for the routine analysis of gentamicin sulphate in pharmaceutical preparations.



Gentamicin Sulphate According to EP & USP Method

Introduction

Gentamicin is a broad spectrum water-soluble antibiotic belonging to the group of aminoglycosides. It is manufactured by a fermentation process and consists of a mixture of related gentamicin components. The main constituents are gentamicin C1, C1a, C2 and C2a. Usually also other minor aminoglycosides are found in a pharmaceutical gentamicin preparation. The number of impurities and components possible makes the chromatographic analysis not quite straightforward.

Because of the presence of a sugar moiety in these analytes the selectivity and inherent sensitivity of pulsed amperometric detection (PAD) is a very attractive approach [1]. The analysis of Gentamicin sulphate in pharmaceutical formulations based on HPLC-PAD is described in the European and U.S Pharmacopoeia [2,3].



Figure 1: ALEXYS analyzer for Gentamicin.

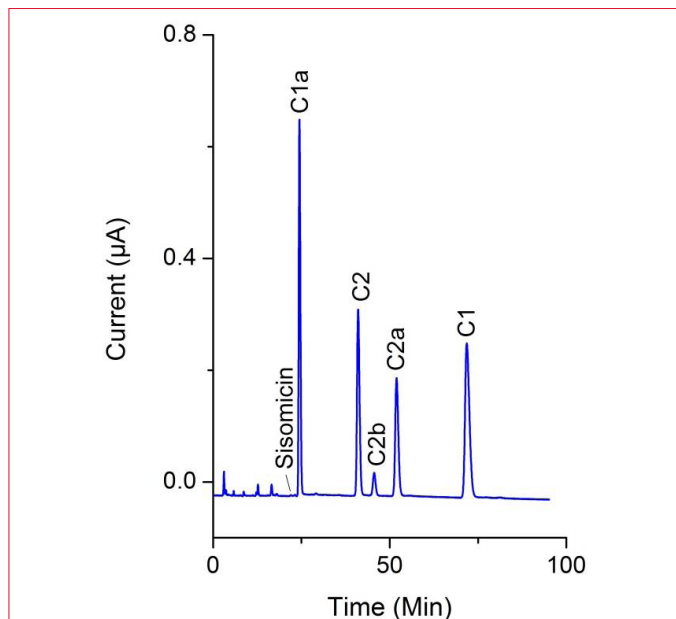


Figure 2: 20 µL injection of a 200 µg/mL Gentamicin sample in mobile phase (Test solution (b) as described in EP and USP monograph).

Method

The method & conditions for separation and detection described in the 2014 EP and USP monograph are almost identical. The monographs differ slightly with respect to system suitability requirements and acceptance criteria for pharmaceutical formulation. In addition, the EP also describes the analysis of related substances (impurities).

In the monographs the use of the following column type is described for the separation of Gentamicin: size 250 mm, ID 4.6 mm, octadecylsilyl silica gel stationary phase (packing L1) and particle size 5 µm. The Phenomenex Luna 5 µm C18(2), 250 x 4.6 mm column which matches this criteria was chosen for the method evaluation. For the detection of Gentamicin PAD is mandatory using an Au working electrode (WE), Ag/AgCl reference electrode (RE) and stainless steel auxiliary electrode (AE). The Antec VT-03 electrochemical flow cell matches these requirements and was used in this evaluation. Note that both column and flow cell are not per se the optimal choice for separation & detection but were chosen to fore fill the USP and EP assay. An alternative approach for the analysis of Gentamicin with significantly shorter analysis time is described in reference [5].



Table 1

LC-EC conditions	
HPLC*	ALEXYS analyzer for Gentamicin with post-column addition kit (375 µL mixer)
Column	4.6 mm ID x 25 cm, 5µm packing L1
Mobile phase	7 mL/L Trifluoroacetic acid, 250 µL/L Pentafluoropropanoic acid, 4 mL/L 12.5M NaOH (carbonate-free) adjusted to pH 2.6, 15.5 mL/L Acetonitrile
Flow rate	1.0 mL/min, post-column: 0.3 mL/min
V _{injection}	20 µL
Temperature	35°C for separation, mixing and detection
Flow cell**	VT-03™ with Au WE, stainless steel AE and Ag/AgCl Salt bridge RE, spacer 50 µm
Potential waveform	E1, E2, E3: +0.05,+0.75, -0.15 V ts, t1, t2, t3: 0.3, 0.4, 0.15, 0.45 s
I-cell	ca. 0.5 µA
ADF	0.5 Hz

*) Note that the presented data are obtained with an older version of the ALEXYS LC system than shown in fig 1. **) Original work was performed with a VT-03, recommended cell: FlexCell with Au WE, HyREF and stainless steel AUX.

The ALEXYS LC-EC Analyzer was equipped with a second pump for the post-column addition of 20 g/L NaOH (carbonate-free). Mixing of the post-column reagent was achieved using a 375 µL PEEK mixing coil.

The mobile phase was prepared as described in the EP & USP monographs (Table 1). The concentration Acetonitrile was slightly adjusted to 15.5 mL/L to optimize the separation. A 3 step waveform was applied with the following settings E1 = +0.05 V, E2 = +0.75 V, E3 = -0.15 V, t1 = 0.4 s, t2 = 0.15 s, t3 = 0.45 and ts = 300ms. The cell current was typical about 0.5 µA with these PAD settings.

The peaks of the Gentamicin main constituents and impurities (A: Sisomicin; B: Garamine) in the recorded chromatogram were identified using the chromatogram supplied with the standard *Gentamicin for peak identification CRS*.

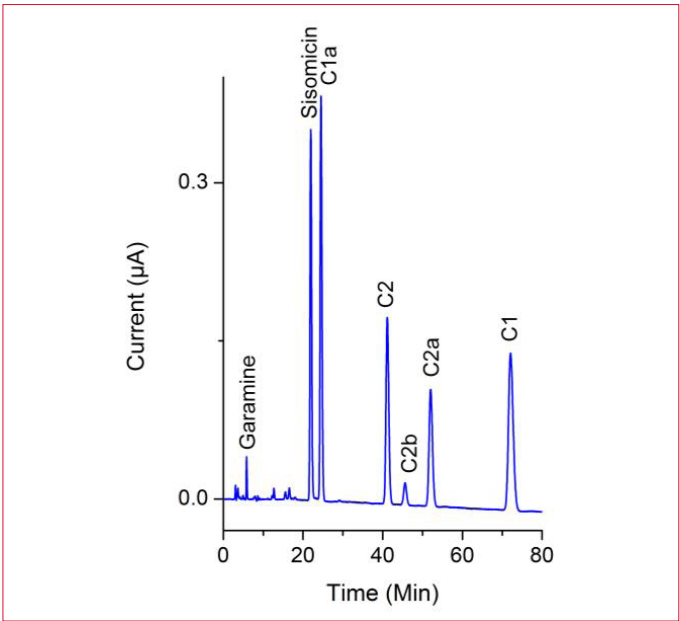


Figure 3: 20 µL injection 20 µg/mL Sisomicin sulphate CRS with 100 µg/mL Gentamicin sample in mobile phase (Reference solution (d) as described in EP monograph).

Table 2

Retention time of Gentamicin main constituents		
Component	Retention time (min)	Relative Retention*
Garamine (Impurity B)	5.8	0.27
Sisomicin (Impurity A)	22.0	1.0
Gentamicin C1a	24.5	1.1
Gentamicin C2	41.1	1.9
Gentamicin C2b	45.6	2.1
Gentamicin C2a	52.0	2.4
Gentamicin C1	72.1	3.3

*) Relative retention time with reference to impurity A (22 min).

Results

System suitability

In the EP monographs for gentamicin sulphate the following system suitability requirement are specified:

- **Resolution:** minimum 1.2 between impurity A and Gentamicin C1a and 1.5 between Gentamicin C2 and C2b in chromatogram obtained with reference solution (d).
- **Signal-to-Noise ratio:** minimum 20 for the principal peak in the chromatogram obtained with the reference solution (c).

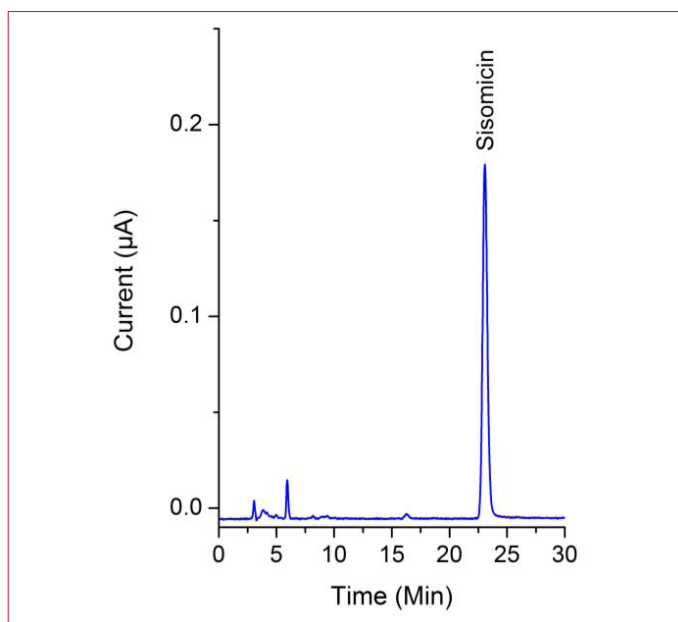


Figure 4: 20 µL injection 10 µg/mL Sisomicin sulphate CRS in mobile phase.

The system suitability was evaluated using the chromatograms of reference solution (c) and (d), see figure 3 and 2 respectively.

Table 3

EP system suitability Requirement		
Parameter	EP criteria	Measured
Resolution between Impurity A & C1a	> 1.2	3.2
Resolution between C2 and C2b*	> 1.5	3.4
Signal-to-Noise ratio (Impurity A)	> 20	323

*) USP requirement: resolution between C2 and C2.

The system suitability requirements are met for all parameters (table 3). Note: in the USP monograph the only system requirement is that the resolution between C2 and C2b is met (> 1.5).

Linearity and repeatability

The linearity of gentamicin was investigated in the concentration range of 25 – 200 µg/mL. For all gentamicin derivatives the correlation coefficients were better than 0.997 for peak areas. The relative standard deviation (RSD) in peak area for a triplicate injection of test solution (b) was ranging between 0.3 – 0.6% for C1, C1a, C2 and C2a. Only for C2b, with its relatively low peak height, the RSD was slightly higher (1.1%). The LOD (S/N ratio of 3) for Impurity A was 9 ng/mL.

Sample analysis

For a commercial sample the composition and related substances were analyzed and evaluated using the EP and USP acceptance criteria. The relative percentage of each gentamicin derivative in the commercial formulation was calculated using the peak area obtained from the chromatogram of test solution (b) shown in figure 1. The sum of all peak areas (C1a, C2 C2a, C2b and C1) corresponds to 100%.

Note that the calculation of the composition for the EP and USP slightly differ. In the EP the sum of C2, C2a and C2b is used; in the USP monograph the sum of C2 + C2a and the sum of C2b + C1. The results are shown in table 4; it is evident that the evaluated commercial sample met the acceptance criteria of both the EP and USP.

Table 4

EP system suitability requirement				
Peak	EP*		USP*	
	Limits (%)	Calculated (%)	Limits (%)	Calculated (%)
C1a	10-30	28	10-35	28
C2	35-55	41	25-55	38
C2a			25-50	34
C2b				
C1	25-45	31		

*) The calculation of the composition for EP and USP slightly differ. In the EP the sum of C2, C2a and C2b is used; in the USP monograph the sum of C2 + C2a and the sum of C2b + C1.

In addition, the EP monograph also describes acceptance criteria for impurity levels in commercial samples. For that purpose all impurities are quantified and compared to the response of the principal peak (Impurity A) obtained from the chromatogram of reference solution (c).

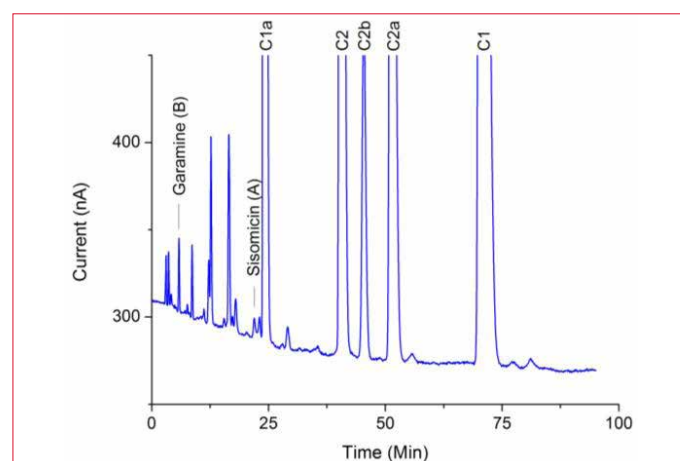


Figure 5: 20 µL injection of test solution (a) for the impurity quantification (1 mg/mL Gentamicin sample in mobile phase).



The relative peak areas of all impurities in the commercial sample are listed in table 5.

Table 5

Impurity analysis		
Impurity	RT (min)	Relative Peak Area*
1	3.1	0.07
2	3.6	0.07
3	4.2	0.02
Garamine (Impurity B)	5.8	0.11
5	7.6	0.02
6	8.7	0.11
7	11.2	0.02
8	12.2	0.13
9	12.7	0.39
10	15.5	0.03
11	16.5	0.50
12	17.3	0.04
13	18.0	0.11
Sisomicin (Impurity A)	22.0	0.08
15	23.1	0.08
17	28.0	0.02
18	29.1	0.09
22	55.6	0.07
24	77.2	0.07
25	81.1	0.11
Total	-	2.11

*) Relative Peak Area of the impurities are calculated in the following way:
 Relative peak area = Area of the impurity divided by the peak area of the principle peak in the chromatogram obtained with reference solution (c).

The EP acceptance criteria for the amount of impurities are:

- **Impurity A, B (and any other impurity):** Not more than 3x the peak area of sisomicin peak in the chromatogram of reference solution (c).
- **Total impurities:** Not more than 10x the peak area of sisomicin peak in the chromatogram of reference solution (c).
- **Discard limit:** Impurities with peak areas smaller than 0.5x the peak area of sisomicin peak in the chromatogram of reference solution (c) can be discarded.

The commercial sample met all impurity acceptance criteria. In fact the response of the majority of all impurities in the sample was under the discard limit of 0.5.

Table 6

Test and reference solutions EP	
Sample solution (a)	1 mg/mL Gentamicin sample in MP
Sample Solution (b)	0.2 mg/mL Gentamicin sample in MP
Reference Solution (a)	0.2 mg/mL Gentamicin for peak identification CRS in MP
Reference Solution (b)	1 mg/mL Sisomicin CRS in MP
Reference Solution (c)	10 µg/mL Sisomicin CRS in MP
Reference Solution (d)	20 µg/mL Sisomicin CRS with 100 µg/mL Gentamicin sample in MP

Conclusion

The ALEXYS analyzer provides a reliable solution for the analysis of the composition & impurities in commercial Gentamicin Preparations following the official methods of the EP and USP.



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Table 7

Reagents and standards	
NaOH 50%, carbonate-free	Boom Chemicals, pn 80011912
Trifluoroacetic acid, HPLC grade	Fischer Scientific, pn T/3258/PB05
Pentafluoropropionic acid, 97%	Acros Chemicals, pn 416920500
Acetonitrile, HPLC grade	Acros Chemicals, pn 268270025
Deionized Water. >18 MΩ-cm	Barnstead, Easy pure II
Gentamicin sulfate CRS, 16500 IU/vial	EP, pn G0200000, batch 8.1
Gentamicin for peak identification CRS*	EP, pn Y0001363, batch 1.0
Sisomicin sulphate CRS, 77.7%	EP, pn S0660000, batch 2.1

*)Gentamicin for peak identification CRS; not injected, reference chromatogram for peak identification downloaded from the following location: <http://crs.pheur.org/db/4DCGI/View=Y0001363>

Ordering information

Recommended ALEXYS analyzer + parts	
180.0058W	ALEXYS Antibiotics base system - Isocratic
180.0605EP	Post Column Kit EP
102.4325EP	Flexcell Au HyREF with stainless steel AUX
250.1045	Flattening/polishing kit for metal WE
184.0209	Glass bottle assembly, 1L, Helium
Column	
00G-4252-E0*	Luna® C18 column, 250 x 4.6 mm ID, 5 µm

*) The column is manufactured and sold by Phenomenex Inc., Torrance, California, USA. Luna is a registered trademark of Phenomenex Inc.

References

1. W.R. LaCourse, "Pulsed Electrochemical Detection in High Performance Liquid Chromatography", John Wiley & Sons, New York, 1ed, 1997.
2. Gentamicin sulphate, *European Pharmacopoeia (EP)*, 8.1, (2014) 2326 -2382
3. Gentamicin sulphate, *United States Pharmacopoeia (USP)*, USP37-NF32, 3138-3139
4. V. Manyanga, K. Kreft, B. Divjak, J. Hoogmartens, E. Adams, *J. Chromatogr. A*, 1189, 347-354 (2008).
5. *Gentamicin Sulphate in pharmaceutical formulations*, Antec application note, 217_013

For research purpose only. The information shown in this communication is solely to demonstrate the applicability of the ALEXYS system. The actual performance may be affected by factors beyond Antec's control. Specifications mentioned in this application note are subject to change without further notice.

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